Brain Asymmetry as a Potential Biomarker for Developmental TCDD Intoxication: A Dose–Response Study

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Previous studies have indicated that *in ovo* exposure to 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) and related compounds is correlated with the development of grossly asymmetric brains. This asymmetry is manifested as a difference between the two halves of the forebrain and the tecta. Previously, only wildlife species (heron, cormorant, and eagle) had been shown to manifest this response. In the wildlife studies, the frequency and degree of left–right interhemispheric differences had been correlated with the levels of polychlorinated dibenzo-*p*-dioxin toxic equivalency factors (TEFs) in eggs from the same nest (heron, cormorant). We studied the effect of *in ovo* exposure to TCDD on the brain throughout development in a sensitive laboratory model (chicken). Embryos from chicken eggs (*Gallus gallus*) injected with one of several doses of TCDD or vehicle control were sacrificed after 9, 11, 13, 15, 17, or 20 days of incubation, or incubated to hatch and then sacrificed either within 24 hr or at 3 weeks post-hatch. Measurements of both chicken embryo and hatchling brains indicated that 1) TCDD alone induced the brain asymmetry in developing chickens; 2) this brain asymmetry was similar to that observed in animals exposed in the wild to a mixture of TCDD-related contaminants; 3) there was a dose-related increase in both the frequency and severity of brain asymmetry observed at all ages measured; and 4) the asymmetry was measurable in embryonic brains at an age when the braincase was a thin, flexible layer (embryonic day 9), implying that the effect of TCDD was directly on the developing brain and not indirectly via an effect on the braincase. *Key words*: asymmetry, brain, development, dioxin, embryo, TCDD. *Environ Health Perspect* 105:718–725 (1997)

Polychlorinated dibenzo-*p*-dioxins (PCDDs), polychlorinated dibenzofurans (PCDFs) and polychlorinated biphenyls (PCBs) are virtually ubiquitous environmental pollutants that are known to bioconcentrate in animal tissue, biomagnify up the food chain, cross the placenta into mammalian embryos, and be deposited into the eggs of egg-laying animals (*1–3*). These compounds are part of the broader class of compounds known as polyhalogenated polycyclic aromatic hydrocarbons (PHAHs), the most acutely toxic of which is 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) (*3*). TCDD in particular, and PCDDs, PCDFs, and PCBs in general, are known to be embryotoxic and teratogenic, causing abnormalities in the palate or beak, heart, and kidney, as well as causing general depression of embryonic growth (*1,4,5*). In addition, these compounds are linked to abnormalities in the development of the nervous system. PCBs, for example, have been linked to changes in the biochemical development of parts of the nervous system and nervous system-related tissue (*6*). PCBs, PCDFs, and PCDDs, as well as other organochlorines, have all been linked to behavioral changes in a wide variety of animals (*2,6–8*). Indications from the biochemical studies, however, are that some of the nervous system effects of these compounds may not be mediated by the laterally substituted, coplanar compounds prototypically represented by TCDD (*6*).

Previous studies from our laboratory have indicated that *in ovo* exposure to TCDD and related compounds is also correlated with the development of brains that are grossly asymmetric (*9–12*). This asymmetry is manifested as differences in several parameters between the left and right halves of the forebrain and is also evident in the tecta, again as a left–right difference. Previously, only wildlife species (heron, cormorant, eagle) have been shown to manifest this response (*9–12*). In the wildlife studies, the frequency and degree of left–right interhemispheric differences had been correlated with the levels of PCDDs and/or TCDD toxic equivalents in eggs from the same nest (heron, cormorant) or in the blood (eagle). As environmentally exposed wildlife species are virtually always exposed to a mixture of compounds, the question remained whether the asymmetry was being induced by the TCDD-like compounds or by some other compounds that coexisted

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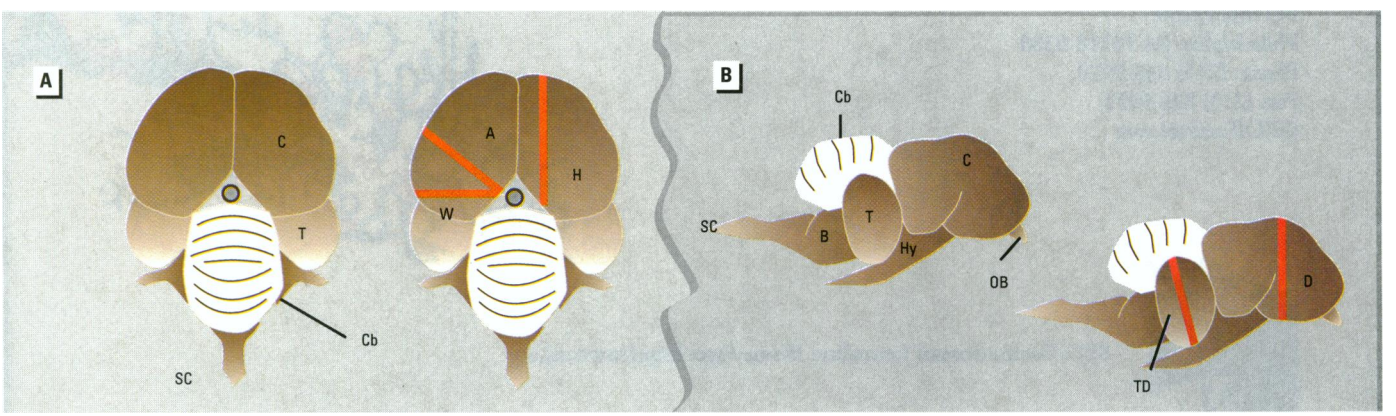

Figure 1. Drawings of the chicken brain from the (A) dorsal and (B) lateral aspects illustrating the major brain regions and the individual measurements made. Abbreviations: A, angle, D, depth; H, height; TD, tectal depth (tectal width not shown); C, cerebrum (telencephalon); T, tectum; Cb, cerebellum; SC, spinal cord; B, brain stem; OB, olfactory bulb; W, width; Hy, hypothalanus.

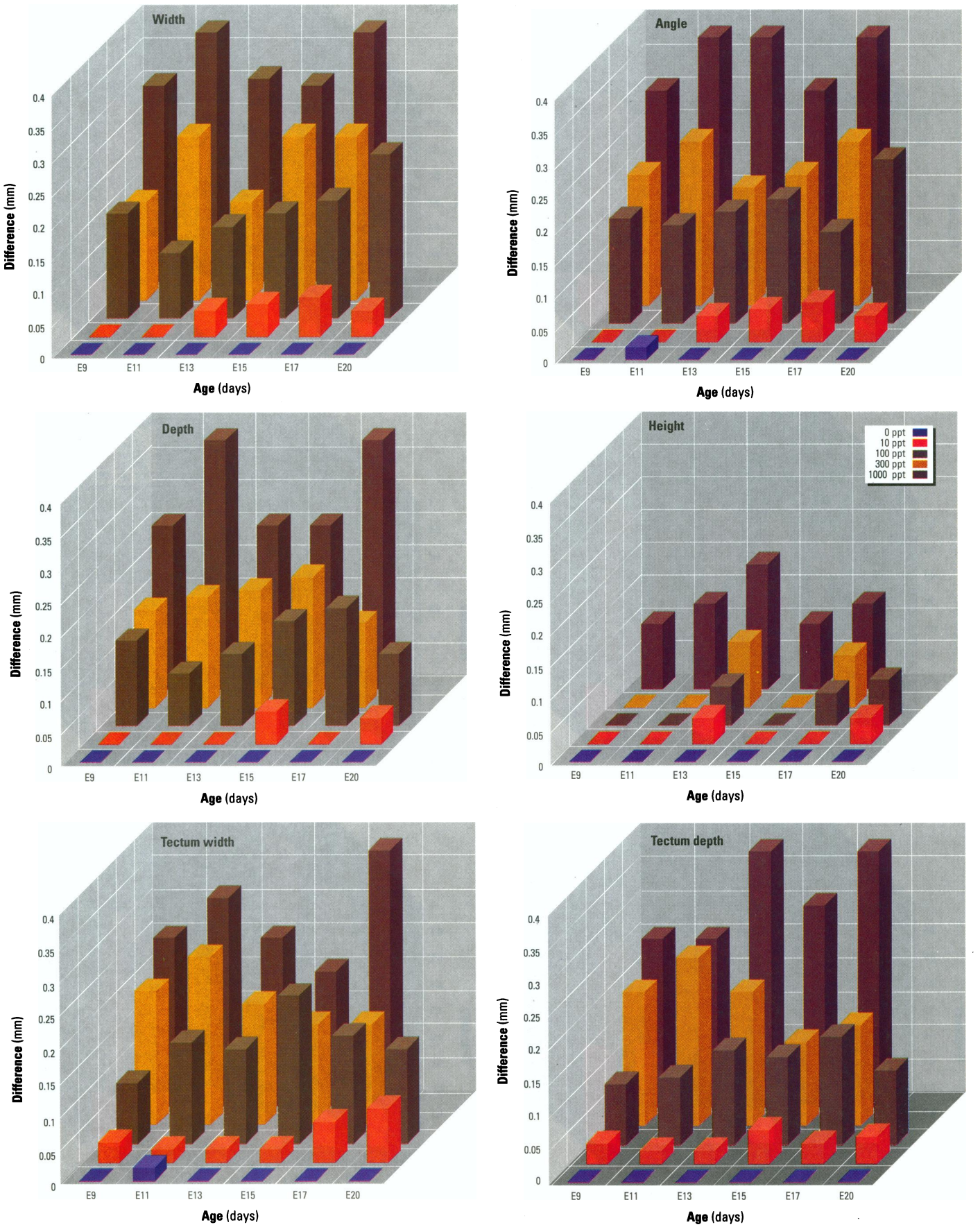

Figure 2. Mean symmetry differences in brain of chicken embryos averaged by age and dose of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin. E, embryonic day or incubation day. Measurements include width, angle, depth, and height of the forebrain and width and depth of the tectum (see Fig.1).

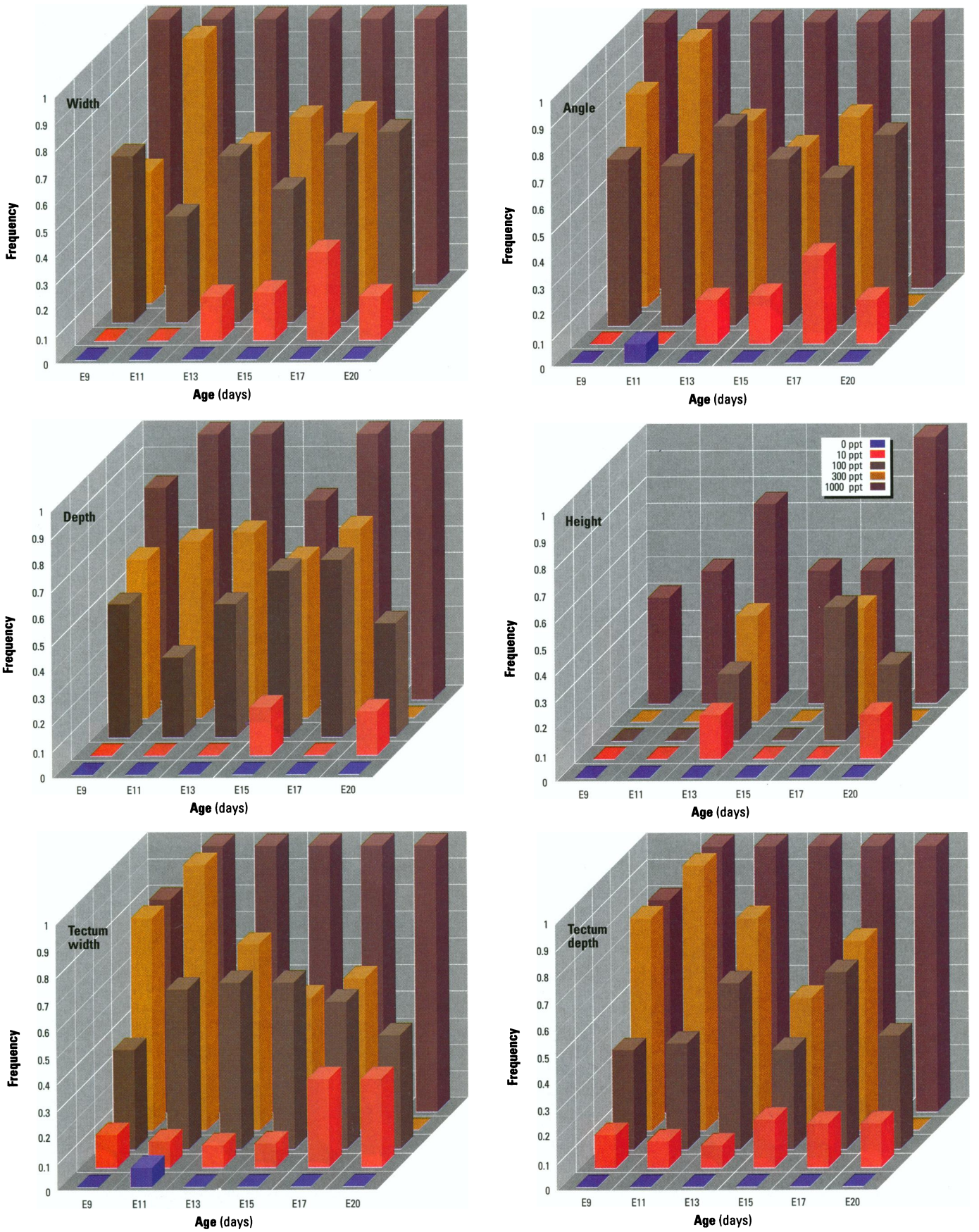

Figure 3. Proportion of asymmetric brains of chicken embryos graphed by age and dose of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin. E, embryonic day or incubation day. Measurements include width, angle, depth, and height of the forebrain and width and depth of the tectum (see Fig.1).

with the TCDD-like compounds in the environment. In addition, there was a question of when during embryonic development the asymmetry began to develop and whether, once developed, the asymmetry remained evident. Finally, it was not clear whether TCDD affected the brain directly or whether the gross brain malformation was induced indirectly via an effect on the braincase. Therefore, we initiated this developmental, dose–response, controlled laboratory study to determine whether TCDD alone could induce brain asymmetries similar to those observed in wildlife species contaminated in the wild with TCDD and TCDD-like compounds.

Methods

Fertile white leghorn chicken (*Gallus gallus*) eggs were injected before the start of incubation with one of several doses of TCDD [10, 100, 300, or 1000 pg TCDD/g egg (= parts per trillion or ppt) for the embryos sacrificed before hatching and 10, 30, 60, 100, 300, or 1000 ppt for the embryos allowed to hatch] or with vehicle control (safflower oil). TCDD was obtained from Ultra Scientific (Providence, RI) predissolved to a known concentration in safflower oil. Injection volumes were normalized to the weight of the individual eggs: 0.1 µl of TCDD or oil per gram of egg was injected through a hole in the shell above the air sac using a Hamilton syringe. The hole in the shell was subsequently sealed with paraffin. The eggs were incubated at 37.5°C dry bulb and 30°C wet bulb (approximately 56% humidity) until sacrifice or hatch. Embryos were sacrificed at embryonic days (E; incubation days) E9, E11, E13, E15, E17, and E20. All eggs were removed from the incubator at the same time of day that they had initially been placed in the incubator. All embryos were sacrificed in random order. Other injected eggs were incubated until hatching, and the hatchlings were sacrificed either within 24 hr of hatching or at 3 weeks post-hatch. Hatchlings raised to 3 weeks were raised in groups including one of each dose, housed by group in 1 1/2 ft (W) × 2 ft (D) × 1 1/2 ft (H) plastic cages, warmed with a 100 (week 1) or 60 watt (weeks 2–3) light bulb. Food and water were provided *ad libitum*. The birds were individually coded with permanent marker on their feathers.

At sacrifice, the older embryos (E13, E15, E17, E20), hatchlings, and 3-week-old birds were all anesthetized with phenobarbital and either perfused through the heart with phosphate buffered saline (PBS, pH 7.4) followed by 4% paraformaldehyde made in PBS (PARA, pH 7.4) or were sacrificed with phenobarbital and then dissected rapidly to remove the brains. The dissected brains were then immersion-fixed in 4°C PARA. Sacrifice method was by injection set across all doses and controls, with injection sets of three birds each per dose and by age of sacrifice. The younger embryos were sacrificed by immersion in cold PARA (4°C). All embryos were stored in PARA at 4°C until shortly before necropsy, when they were transferred to PBS (4°C) with 0.625% sodium azide added to prevent mold growth. All animals were handled following protocols approved by the University of British Columbia (UBC) Animal Care

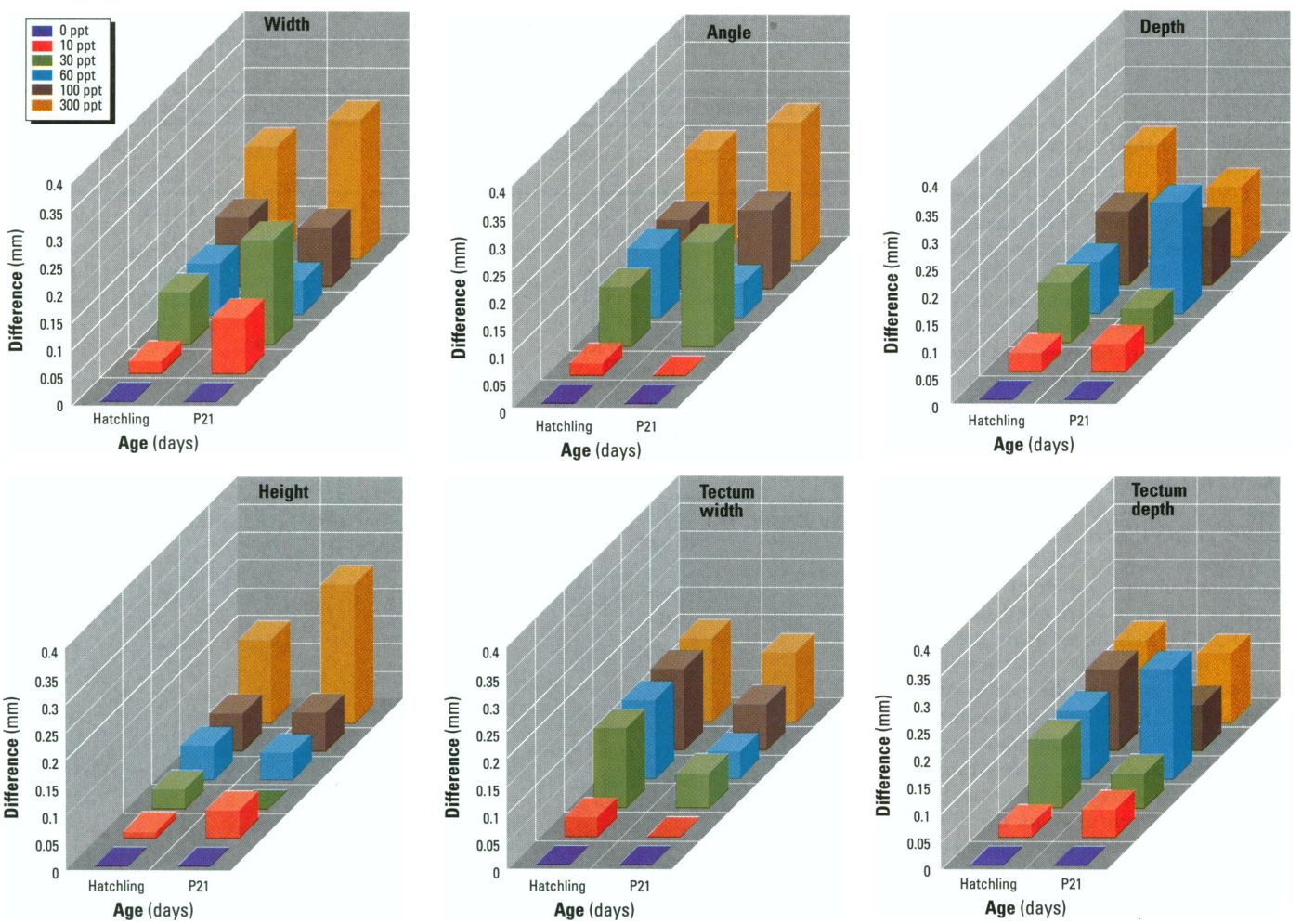

Figure 4. Mean symmetry differences in brain of post-hatch chicks averaged by age and dose of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin. P21, postnatal day 21. Measurements include width, angle, depth, and height of the forebrain and width and depth of the tectum (see Fig.1).

Committee, and handling of hazardous materials was approved by the UBC Occupational Safety and Health office.

Six measurements were made on each brain, four on the forebrain region and two on the tecta. The tectal depth and forebrain depth were measured from the lateral aspect of the brain, while the tectal width was measured from the ventral aspect of the brain. The other three measurements (height, width, angle) were all made from the dorsal aspect of the forebrain. The four forebrain measurements (illustrated in Fig. 1) were mediolateral width just rostral to the pineal (width), mediolateral measurement from the same central point just rostral to the pineal and out to the lateral edge at a 35° angle from the horizontal defined by the width measurement (angle), rostral–caudal length from mid-wulst (the dorsorostral forebrain protrusion) to the caudal-most part of the forebrain on that rostrocaudal axis (height), and the dorsoventral depth of the brain at the point where the hypothalamus meets the telencephalon (at the level of the pre-optic area, depth).

Brains were measured as described previously (*7,8*), except that the tecta were also measured for this study. Briefly, each measurement was made using a ruler propped parallel to the surface of the brain. The eye was centered over the center of the brain for the width and angle measurements and over the center of the area being measured for the other measurements. All measurements were made two times. If the measurements did not agree, each measurement was made an additional three times and the results were averaged. The measurements reported here represent the difference between left and right halves of the brain and are all presented as measurements from the left side of the brain minus measurements from the right side of the brain.

We regressed each measurement on TCDD concentration and on age using the Regression and General Linear Models procedures in SAS (PROC REG, PROC GLM, SAS Institute, Cary, NC). Significance was determined using a p-value of 0.05. Probit analysis was also performed using SAS (PROC PROBIT) to estimate the ED_{10}s (the dose at which a 10% population response is elicited) and the median effective doses (ED_{50}s) for the observed asymmetry. The standard deviation (SD) reported for the ED_{50} is the standard deviation (σ) reported by SAS for the average (the μ or the ED_{50}). No such values were given for the ED_{10}s by SAS; therefore, no SDs were reported for the ED_{10}s. Further, 95% fiducial limits are not incuded in this paper as they were inconsistently reported by SAS, depending on the sample sizes for each age and dose.

Results

TCDD clearly affected both the frequency and the degree of brain symmetry in a dose-dependent manner (Fig. 2–5). To give an indication of relative variability in the data, the sample size and standard error of the means are listed in Table 1 for the angle measurement (Fig. 2 and 4). Noticeably, the TCDD-induced forebrain asymmetry was only evident at the lowest dose (10 ppt) in the E13 and older animals. However, the tectal asymmetry was already present in the brains exposed to the lowest dose of TCDD at E9. It is also clear from these data that the asymmetry persisted to at least 3 weeks post-hatch, through a period of rapid post-hatching growth. Similarly, although the forebrain measurements were only made from the dorsal aspect, the brain asymmetry was noticeable from both the dorsal and the ventral aspects of the brain (Fig. 6). Table 2 lists the PROBIT determined ED_{10}s and ED_{50}s by age and measurement.

Of the four forebrain measurements, width, angle, depth and height, the first three had the highest R^2 values when regressed against age and TCDD concentration (see equations below). The height measurements had the lowest R^2 values of all of the measurements, in general. The R^2 values for the two tectal measurements tended to be intermediate between the width, angle, and depth measurements and the height measurement, although the R^2 for tectal depth was similar to the R^2 for the width, angle, and depth measurements. When evaluated by age and TCDD concentration, all six measurements were significant at the 0.0001 level. The regression equations (Eq. 1–6) in the shaded box describe the relationships between each measurement, TCDD, and age; p-values for the individual parameters within each equation are listed below each parameter.

Discussion

TCDD injected at the start of incubation clearly induces brain asymmetry in chicken embryos in a dose-dependent manner. The asymmetry is manifested as a consistent left–right hemispheric difference in both the forebrain and the tectum and is present after the initial formation of these brain regions.

Chickens have a 21-day incubation period. Chicken brains (and avian brains in general) do not develop evenly throughout development (Fig. 7). During early incubation (organogenesis), the chicken

Table 1. Standard error of the mean and sample size for angle difference measurements[a]

	Dose (ppt)						
Age	0	10	30	60	100	300	1000
E9	0 (9)	0 (8)	ND	ND	0.13 (8)	0.11 (3)	0.11 (5)
E11	0.07 (14)	0 (10)	ND	ND	0.13 (10)	0 (3)	0.14 (4)
E13	0 (13)	0.1 (12)	ND	ND	0.12 (8)	0.12 (10)	0.14 (4)
E15	0 (15)	0.1 (11)	ND	ND	0.18 (8)	0.2 (9)	0.14 (4)
E17	0 (13)	0.13 (6)	ND	ND	0.18 (9)	0.35 (7)	0.18 (2)
E20	0 (8)	0.1 (6)	ND	ND	0.2 (7)	NS	(1)
P0	0 (22)	0.07 (22)	0.13 (7)	0.13 (8)	0.13 (18)	0.11 (5)	NS
P21	0 (5)	0 (5)	0.24 (4)	0.06 (4)	0.13 (7)	0 (2)	NS

Abbreviations: E, embryonic day or incubation day; P, postnatal day; ND, not done; NS, no survivors.
[a]Values in parentheses are sample size.

Forebrain width difference

0.023249 mm + 0.000372 mm/ppt•TCDD + 0.001665 mm/day•age ($p \leq 0.0001$, $R^2 = 0.3303$) (1)
(0.1855) (0.0001) (0.0525)

Forebrain angle difference

0.038255 mm + 0.00379 mm/ppt•TCDD + 0.000919 mm/day•age ($p \leq 0.0001$, $R^2 = 0.3374$) (2)
(0.0293) (0.0001) (0.2796)

Forebrain depth difference

0.015913 mm + 0.000328 mm/ppt•TCDD + 0.001476 mm/day•age ($p \leq 0.0001$, $R^2 = 0.3277$) (3)
(0.2950) (0.0001) (0.0435)

Forebrain height difference

-0.013928 mm + 0.000147 mm/ppt•TCDD + 0.001609 mm/day•age ($p \leq 0.0001$, $R^2 = 0.1751$) (4)
(0.1841) (0.0001) (0.0014)

Tectal width difference

0.055878 mm + 0.000289 mm/ppt•TCDD - 0.000126 mm/day•age ($p \leq 0.0001$, $R^2 = 0.2583$) (5)
(0.0008) (0.0001) (0.8765)

Tectal depth difference

0.029544 mm + 0.000321 mm/ppt•TCDD + 0.000996 mm/day•age ($p \leq 0.0001$, $R^2 = 0.3239$) (6)
(0.0521) (0.0001) (0.1747)

brain first segments into the prosencephalic, diencephalic, mesencephalic, and rhombencephalic regions (listed from rostral to caudal). By E3–E4, the two forebrain hemispheres (the telencephalon) bubble out in the prosencephalic region of the neural tube. By E4, the mesencephalon begins to develop as an enlarging central structure. The partitioning of the mesencephalon into two (left and right) tectal prominances begins about E5. By E7 or E8, the dominant outgrowths of the brain are the tecta, caudal to the telencephalic outgrowths. The telencephalic hemispheres begin to appear more prominent around E9 or E10 so that, by E12, the forebrain hemispheres are almost equal in size to the tectal outgrowths. By E16 or E18, the forebrain hemispheres have begun to dwarf the more caudal tecta (*13,14*).

Given the difficulty of dissecting out early embryonic brains, we only made asymmetry measurements on brains from E9 embryos and older. By this age, even though the telencephalic hemispheres are still very small and undeveloped, there is clear forebrain asymmetry induced by the higher doses of TCDD used and tectal asymmetry at all doses of TCDD used. Once the forebrain hemispheres begin to develop again, in the latter half of the 21-day incubation period, the brain asymmetry is also manifested at even the lower doses of TCDD. Once the brain has started to develop asymmetrically, evidence from the brains of the hatchling and 3-week-old birds indicates that the brains remain asymmetrical. Thus, the asymmetry endpoint appears to be increased in sensitivity following and during periods of rapid development of the brain regions affected (forebrain and tecta). Once the asymmetry is present, the brain appears to continue to grow such that the asymmetry remains. This implies that TCDD may be affecting the brain during periods of high mitogenic activity and may be differentially inducing increased mitogenesis on the two sides of the brain. The ED_{10}s (Table 2) confirm this increase in sensitivity in the forebrain measurements at the time of the forebrain expansion and indicate that the sensitivity of the tectal measurements are already maximal by E9 (posttectal expansion).

These results also suggest that TCDD affects primarily the brain and not primarily the braincase, with a secondary effect on the brain. At E9, the earliest that we have attempted to measure the brain asymmetry, the braincase is a very thin, almost membrane-like covering over the brain. At this point in development, the braincase is very flexible and appears to present little or no resistance to the brain developing underneath. Thus, while there may also be a direct effect of TCDD on braincase development (which we have not in any way attempted to assess), our results indicate that there is a direct effect of TCDD on the brain as it undergoes rapid development.

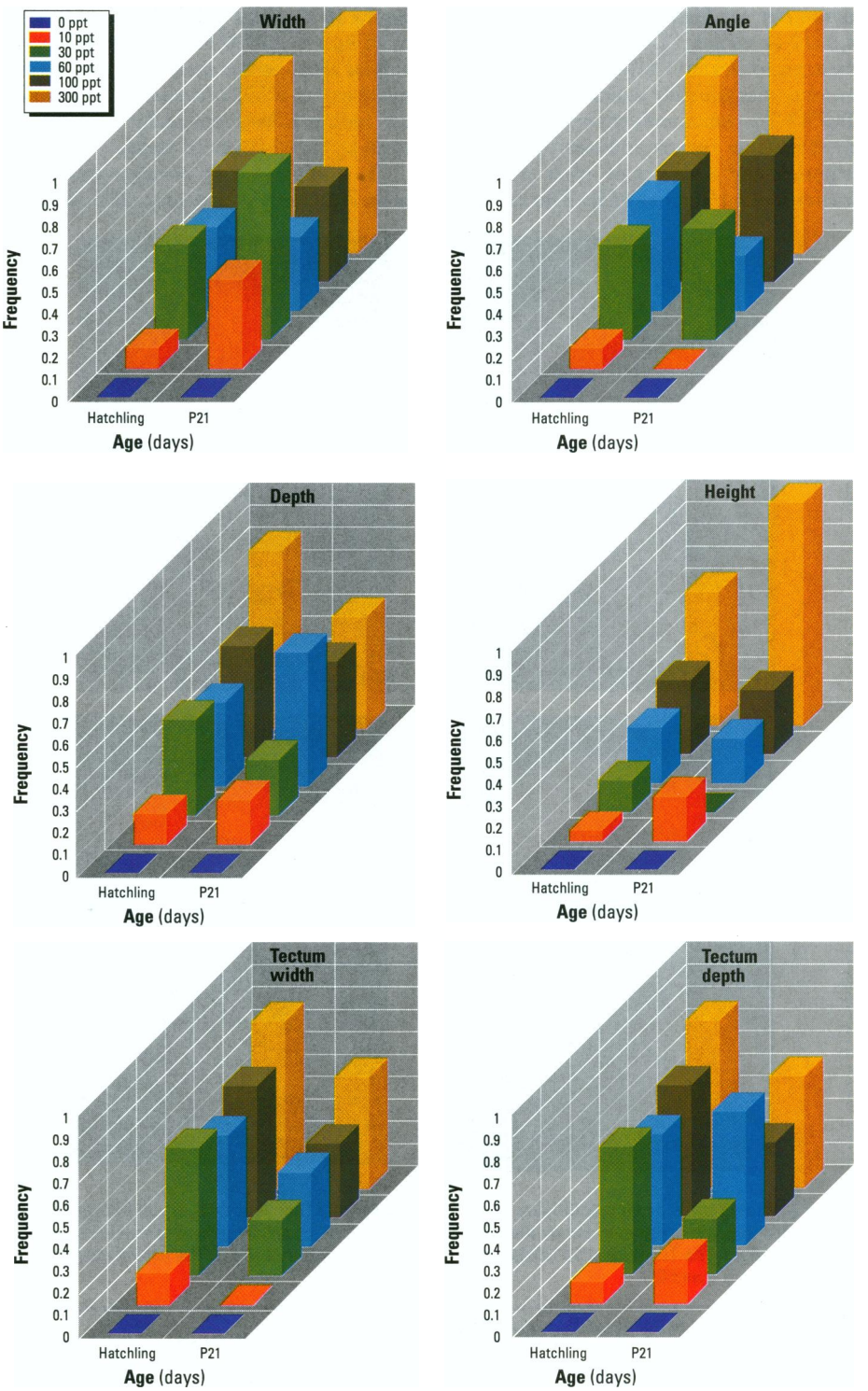

Figure 5. Proportion of asymmetric brains in post-hatch chicks graphed by age and dose of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin. P21, postnatal day 21. Measurements include width, angle, depth, and height of the forebrain and width and depth of the tectum (see Fig.1).

These results in TCDD-injected chickens are consistent with observations we have previously made on several wildlife species (great blue herons, double-crested cormorants, and bald eagles) exposed *in ovo* in the wild to a mixture of TCDD-related chemicals, including (depending on the study) PCDDs, PCDFs, and PCBs (*10–12*). In each of these studies, the brain

asymmetry was highly and significantly correlated to either TCDD levels, TCDD-toxic equivalents (TEQs), or some other measure that generally indicates exposure to TCDD-like compounds ethoxyresorufin *O*-deethylase, an enzyme induced by TCDD exposure and a variety of other environmental contaminants) (*15*). The present results in chickens confirm that although other non-TCDD-like congeners may have had an effect on the measured brain asymmetry in the wildlife studies, TCDD alone can induce a developmentally linked gross brain asymmetry in avian brains.

It is important to correlate this gross brain asymmetry with functional changes in brain-controlled activity. At this time we do not know specifically which brain-mediated functions (if any) may be affected by this gross dysmorphism, although behavioral studies are ongoing. From previous histological studies we know that the pyriform cortex of the heron hatchlings from a PCDD- and PCDF-contaminated colony was increased in both mediolateral width and cell density (*9*). The pyriform cortex is associated with the limbic system, a series of structures in the brain that affect emotion and instinctive behavior. In addition, the pyriform cortex is the main cortical area involved in olfactory discrimination and is believed to receive indirect input from the olfactory bulb (*16*). Olfaction is also generally believed to affect instinctive behaviors. We did not, however, track sidedness in the brains for the initial heron studies and, given how the brains were processed, we can not determine sidedness after the fact. In addition, the pyriform cortex is a thin strip on the outside of the forebrain. The relatively large differences in the left–right hemispheres seen in this study could not be totally attributed to changes in such a thin strip of tissue, nor could any changes in the tecta be directly attributed to changes in the thickness of the pyriform cortex. Future studies are needed to determine which nuclei (an amalgamation of functionally and/or anatomically related neurons) and biochemical markers in the brain are specifically affected by TCDD.

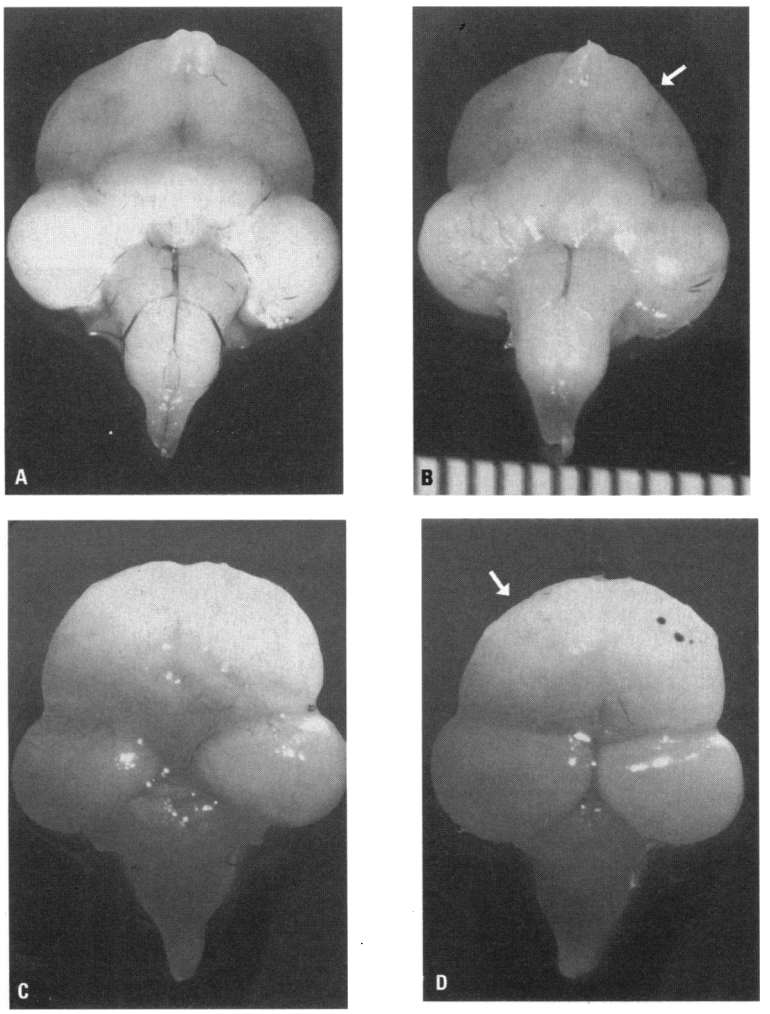

Figure 6. Photographs of embryonic day 13 chicken brains from the ventral (A, B) and dorsal (C, D) aspects illustrating the flattened arc on the left side of the brain (B, D; indicated by arrows) due to *in ovo* (TCDD) exposure. The TCDD injected chicken brain (300 ppt) is shown in B and D. The noninjected control brain is shown in A and C.

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Table 2. Probit (log_{10}) determined ED_{10}s and ED_{50}s by age and measurement

	Height		Width		Angle		Depth		Tectal Width		Tectal Depth	
Age	ED_{10}[a]	ED_{50}[b]	ED_{10}	ED_{50}	ED_{10}	ED_{50}	ED_{10}	ED_{50}	ED_{10}	ED_{50}	ED_{10}	ED_{50}
E9	832.52	1046.20 ± 1.2	22.77	107.09 ± 3.35	24.85	89.32 ± 2.71	21.00	168.68 ± 5.08	8.75	127.32 ± 8.08	8.75	127.32 ± 8.08
E11	803.38	1000.00 ± 1.19	85.36	103.98 ± 1.17	77.56	95.89 ± 1.18	53.96	164.73 ± 2.39	11.64	58.26 ± 3.51	14.62	87.53 ± 4.04
E13	5.35	477.26 ± 33.23	4.97	82.07 ± 8.92	4.83	56.38 ± 6.8	32.27	131.63 ± 2.99	11.91	93.13 ± 4.98	12.39	79 ± 4.24
E15	806.74	1000.00 ± 1.18	6.12	67.83 ± 6.54	4.72	67.77 ± 8.0	2.71	85.15 ± 14.73	10.02	90.93 ± 5.59	6.41	110.25 ± 9.21
E17	44.07	496.50 ± 6.62	0.98	34.91 ± 16.3	1.20	47.93 ± 17.78	15.34	106.38 ± 4.53	0.58	62.51 ± 38.52	6.37	58.9 ± 7.12
E20	7.21	241.29 ± 15.47	6.44	42.3 ± 4.34	6.44	42.3 ± 4.34	6.37	111.17 ± 9.31	0.58	90.65 ± 51.3	4.76	111.17 ± 9.31
P0	22.70	247.17 ± 6.44	9.67	86.83 ± 5.54	9.12	78.78 ± 5.38	6.85	79.03 ± 6.74	4.93	58.71 ± 6.91	7.16	62.28 ± 5.41
P21	15.80	196.33 ± 7.14	0.029	33.75 ± 250.16	16.05	70.69 ± 3.18	2.16	206.97 ± 35.14	18.35	199.72 ± 6.44	1.23	195.93 ± 52.18

Abbreviations: E, embryonic day or incubation day; P, postnatal day; ED_{10}, effective dose eliciting a 10% response; ED_{50}, median effective dose.
[a]No standard deviation (SD) is given by SAS (SAS Institute, Cary, NC) for the ED_{10}s, therefore no SD is listed.
[b]μ (ED_{50}) ± σ (SD).

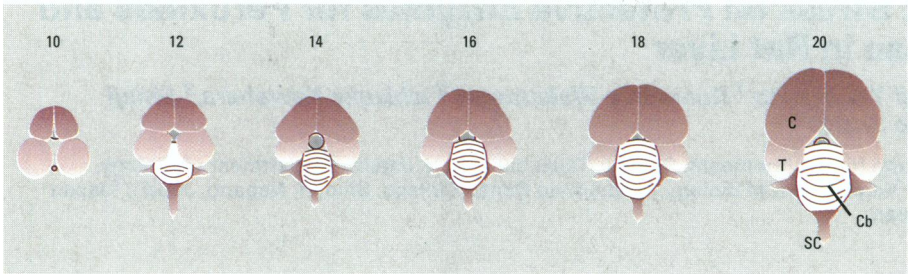

Figure 7. Drawings of the chicken brain from the dorsal aspect between embryonic days 10 and 20, just prior to hatching. Abbreviations: C, cerebrum; T, tectum; Cb, cerebellum; SC, spinal cord. Modified from Romanoff (*14*).

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OCTOBER 26–30 1997

HILTON HEAD, SOUTH CAROLINA

SHORT COURSES

Four half-day short courses will be offered, two in the morning and two in the afternoon.

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(1) Disposition of Biotechnology Compounds
- Peptides, Proteins, Oligomers
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- Methods to Evaluate Combinational Libraries

(2) Risk Assessment and Safety Evaluation
- Chemicals in the Environment
- Pharmaceuticals and Foods
- Risk Management and Regulation

ADDITIONAL TOPICS INCLUDE:
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- Chemistry and enzymology of biotransformation
- Drug interactions
- Alternatives to animal models
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- Physiological barriers
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